Mutations in the neutrophil elastase gene in cyclic and congenital neutropenia

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Severe neutropenia disorders are characterized by extremely low levels of peripheral blood neutrophils, a maturation block of bone marrow progenitor cells and recurring severe bacterial and fungal infections. Recent reports indicated that severe neutropenia is a consequence of an impaired survival and abnormal cell cycle progression of myeloid progenitor cells in both cyclic and severe congenital neutropenia. Mutations in the neutrophil elastase gene were identified in all patients with cyclic neutropenia and most of the patients with severe congenital neutropenia. We hypothesize that expression of mutant neutrophil elastase protein results in deregulation of intracellular activity and premature cell death of myeloid-committed progenitor cells in these disorders, resulting in the lack of peripheral blood neutrophils. The potential molecular mechanisms of mutant-protein-mediated neutropenia is discussed.

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Current Opinion in Immunology 2001, 13:535–538

0952-7915/01/$ – see front matter

Abbreviations

ANC	absolute neutrophil count
CN	cyclic neutropenia
G-CSF	granulocyte-colony-stimulating factor
NE	neutrophil elastase
SCN	severe congenital neutropenia
WAS	Wiscott–Aldrich syndrome

Introduction

The recent identification of mutations in the human neutrophil elastase (NE) gene in patients with inherited and acquired (sporadic) cyclic neutropenia (CN) and severe congenital neutropenia (SCN) provides new insights to the potential mechanism of these disorders and raises many research questions. This discovery attracted the attention of many investigators and has intensified molecular and biochemical studies of the pathogenesis of severe neutropenia. CN and SCN are hematopoietic disorders characterized by extremely low levels of circulating neutrophils in peripheral blood [1–3]. The patients usually suffer from recurring episodes of severe bacterial and fungal infections. Patients with SCN usually have an absolute neutrophil count (ANC) below 0.2×10^9/l, whereas in CN the ANC oscillates from normal or near-normal levels (2.0×10^9/l) to extremely low level (often 0) with 21-day periodicity. Oscillations of other blood elements also occur in CN, but may go unnoticed.

CN is inherited exclusively as an autosomal-dominant disorder [4–6], whereas both autosomal-dominant and autosomal-recessive inheritance has been reported in SCN [7,8]. Long-term treatment with recombinant granulocyte-colony-stimulating factor (G-CSF) increases the level of circulating neutrophils and reduces the frequency of bacterial and fungal infections for both CN and SCN patients [9]. In this review, we describe recent cellular and molecular studies of these conditions and outline potential mechanisms of the pathogenesis of these disorders.

Cellular studies

The lack of neutrophils in CN and SCN is generally attributed to a decreased production of these cells in the bone marrow and reduced delivery of them to the peripheral circulation. Analysis of bone marrow morphology reveals a predominance of immature hematopoietic progenitor cells and an absence of myeloid precursor cells beyond the promyelocytic stage of differentiation in SCN [10,11•]. During the severe neutropenia periods in CN, the bone marrow looks similar to SCN, with very few maturing cells of the myeloid lineage.

Colony-forming assays of CN bone marrow aspirates taken at different points of the neutropenia cycle also reveal oscillations in the clonogenic activity of hematopoietic progenitor cells [12,13]. Examination of clonogenic activity of bone-marrow-derived $CD34^+$ primitive progenitor cells demonstrates a significantly reduced myeloid compartment in the marrow of CN and SCN patients compared with healthy volunteers [14••,15]. We reported that the decreased production of myeloid-committed progenitor cells is due to impaired survival of these cells in CN [14••]. Regardless of when during the neutropenia cycle the bone marrow aspirates were obtained, the bone marrow progenitor cells of the granulocytic lineage, including primitive $CD34^+$ cells, myeloid-committed $CD33^+$ cells, and more-differentiated $CD15^+$ neutrophil precursors, exhibited extremely poor survival characteristics when compared with respective control populations [14••]. Accelerated apoptosis of bone marrow progenitor cells was also observed in all SCN patients studied [15].

It is important to note that the survival characteristics of freshly isolated bone marrow cell subpopulations from CN and SCN patients were not different from those of healthy volunteers. This may be explained by highly efficient removal of apoptotic cells in the human bone marrow by scavenger macrophages as well as the removal of apoptotic cells during the purification process. Accelerated apoptotic cell death in CN and SCN was noted after short-term (usually overnight) incubation of isolated cells in the presence of 10% autologous serum [14••,15]. Cross-incubation of patients' bone marrow cells with control serum (i.e. from

healthy volunteers), or of control cells with patients' serum, and subsequent apoptosis analysis did not suggest a presence of serum-soluble factor capable of inducing apoptosis [14••]. Nevertheless, it is yet possible that the concentration of such a pro-apoptotic factor in 10% autologous serum is not high enough to trigger apoptotic cell death. Further studies are needed to clarify these issues. At any rate, the accelerated apoptosis of bone marrow myeloid-committed progenitor cells appears to be the cellular mechanism leading to low production of granulocytic progenitor cells in the marrow and the lack of neutrophils in the peripheral circulation [16].

Molecular studies

The identification of multigenerational families containing multiple family members diagnosed with CN allowed us to initiate PCR-linkage analysis and positional cloning in order to elucidate the molecular defect of this disorder. In the early stages of this study, three genomic regions with possible candidate genes were excluded [17]. Later, with the availability of a genome-wide scan and automated genotyping using fluorescently labeled DNA markers and the ABI Prism technology (Perkin Elmer, Foster City, CA), a more sophisticated and comprehensive analysis was performed, which resulted in the identification of mutations in the NE gene as a cause of CN [18••]. All patients with inherited or acquired CN exhibited heterozygous substitution, deletion or insertion mutations in the NE gene, suggesting a dominant-negative effect of a mutant NE gene product. On the other hand, the homozygous deletion of the murine NE gene did not result in an SCN-like phenotype, although an increased susceptibility of these mice to infections was reported [19]. These observations imply that NE mutations identified in CN may lead to a gain-of-function rather than to a dominant-negative effect.

Because of similarities such as the reduced production and accelerated apoptosis of bone marrow myeloid progenitor cells in CN and SCN, sequencing analysis of the NE gene in SCN was performed, which resulted in the identification of NE mutations in 43 out of 46 SCN patients examined [20••,21]. The heterozygosity of all SCN-specific mutations identified to date in patients with and without a family history of SCN suggests an autosomal-dominant mode of inheritance. Studies are in progress to determine if mutations of the NE gene are responsible for the cause of autosomal-recessive SCN, or Kostmann syndrome [7].

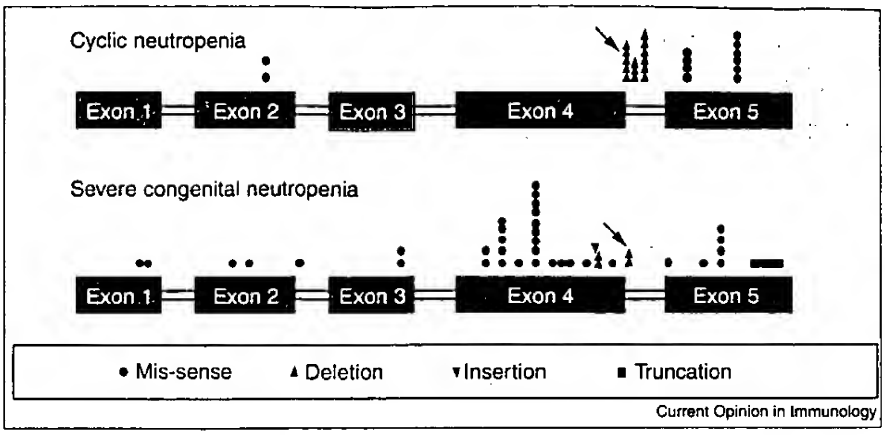
Following the original publications on mutational analysis in CN and SCN, a number of investigators confirmed and reported mutations in the NE gene in congenital neutropenia [22–24]. Studies of neutropenic patients involved in the French Registry revealed mutations of the NE gene in all familial cases of CN and SCN [22], whereas others reported the absence of NE mutations in familial SCN [24]. Interestingly, mutations in exon 1 of the NE gene were reported by Bellane-Chantelot *et al.* [22], implying that mutations in any of the five exons of the NE gene may lead to severe neutropenia phenotype. To date, approximately 7% (3 out of 46) of SCN patients are negative for NE mutations [21], which suggests that (an)other gene(s) may be involved in the etiology of SCN. Recently, it was reported that a substitution mutation that results in constitutive activation of the Wiscott–Aldrich syndrome (WAS) gene product — which is associated with WAS — is a cause of X-linked familial SCN [25••]. It seems possible that, in this case, destabilization of the cellular cytoskeleton by constitutively activated WASP may impair production, and/or result in poor survival, of myeloid-committed progenitor cells.

Figure 1 depicts the genomic organization of the NE gene, which consists of five exons and four introns, spanning approximately 5000 basepairs of genomic DNA; the positions of substitution, deletion, truncation and insertion mutations identified in CN and SCN patients are shown. In CN, these mutations seem to be clustered in intron 4 and exon 5, whereas in SCN the mutations are spread throughout the entire gene. It is puzzling that the same NE mutations have been observed in patients diagnosed as having CN or SCN (Figure 1, arrows). This may be explained by the need for extended periods of serial blood counts to diagnose CN, whereas SCN is often diagnosed on the basis of fewer counts and the characteristic finding of maturation arrest at the promyelocyte stage in the bone marrow. Long-term follow-up of some patients with severe neutropenia also have revealed that the same individual in different periods of his life may have periodic oscillations of ANC in peripheral blood typical of CN and may have continuously low levels of circulating neutrophils that are characteristic of the SCN phenotype at other times. These clinical and genetic findings thus blur the differences between the conditions.

NE belongs to a family of hematopoietic serine proteases that are expressed in bone marrow progenitor cells of the granulocytic lineage and includes proteinase 3, cathepsin G and azurocidin [26]. NE is expressed relatively early during hematopoiesis — at the promyelocyte stage — and is turned off in terminally differentiated neutrophils [27,28]. Normally, the NE protein is processed through the Golgi apparatus, the propeptides are cleaved and the proteolytically active mature NE is stored in azurophil granules until released at sites of inflammation [29]. The NE protein has two *N*-glycosylation sites and four disulphide S–S bridges. Molecular modeling and three-dimensional analysis of NE tertiary structure with positions of NE mutations suggest that, in SCN, the mutations may alter the glycosylation/deglycosylation processing and result in aberrant subcellular localization of mutant NE. Mutations seen in CN seem to affect the resistance of NE to serine protease inhibitors and lead to altered substrate specificity. Exposure of proteolytically active mutant NE to a new range of substrates may lead to premature degradation of vital proteins, abnormal intracellular events and apoptotic cell death. It is also possible that mutant NE hydrolyses gene products that are responsible for recognition of specific microbial components and thus contributes to increased susceptibility to bacterial and fungal infections.

Figure 1

Genomic structure of the NE gene, showing the positions of mutations in CN and SCN patients. Note that some mutations occur at the same position in both conditions (indicated by the arrows).



Future studies focused on determination of subcellular localization of mutant NE and its enzymatic activity will provide critical information on the possible mechanism of mutant-NE-mediated severe neutropenia. We have recently observed accelerated apoptosis of human premyelomonocytic U937 progenitor cells upon transient expression of mutant NE cDNA, similar to bone marrow progenitor cells in CN and SCN patients. Survival of U937 cells transfected with intact NE cDNA was normal and not different from that of mock-transfected cells (AAG Aprikyan, unpublished data). Recently, it was reported that mutant NE transiently expressed in murine myeloid 32D cells and rat basophilic RBL cells did not affect their survival characteristics [30]. This report emphasizes the importance and necessity of lineage- and tissue-specificity studies of constitutive expression of human NE.

Conclusions

The identification of gain-of-function mutations in the NE gene has been the initial step in the elucidation and characterization of the molecular events underlying the pathogenesis of CN and SCN. In the next few years, more comprehensive investigations of the subcellular localization of mutant NE and its interaction with intracellular and extracellular proteins will help to unravel the molecular mechanisms leading to congenital disorders of severe neutropenia and to develop new therapeutic agents capable of reversing the abnormal phenotype.

Acknowledgements

This work was supported by National Institutes of Health grant 2RO1-DK-18951 (to DCD), American Cancer Society Institutional Grant 91-020-06-IRG (to AAGA), and a Research Grant from Amgen, Inc., Thousand Oaks, CA (to AAGA).

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